

## BSSD 2019 Performance Metric Q2

**Goal: Develop metagenomics approaches to assess the functioning of microbial communities in the environment.**

**Q2 Target: Report on ‘omics’-based techniques used to describe microbial activity in the environment.**

### **Executive summary:**

The LANL SFA in Terrestrial Microbial Carbon Cycling aims to inform climate modeling and enable carbon management in terrestrial ecosystems by discovering widespread biological processes that control carbon storage and release in temperate biome soils (primarily arid grass/shrub lands and forests).

Until recently the SFA strategy focused on cataloguing microbial (fungal and bacterial) activity in response to perturbations—physico-chemical drivers of ecosystem change—in field studies led by external collaborators. Perturbations were levels of atmospheric CO<sub>2</sub>, ozone, nitrogen deposition, temperature, precipitation, and/or physical disturbance. “Activity” has been assessed as significant differences between communities, taxa, or functional genes in comparison samples. Lists of active taxa/genes can be referenced against a suite of supporting databases to infer microbial processes that are affected by ecosystem change, but supporting databases are not yet mature enough to effectively achieve this goal. To address this gap, the SFA performed auxiliary studies to acquire reference data describing activities or the functional potential of specific organisms. Studies included use of <sup>13</sup>C-stable isotope probing to identify cellulose-consuming organisms in different soils [1]; biochemical assays of hundreds of Actinobacterial and fungal isolates cultivated from field site samples [6, 7]; genome sequencing of select isolates [8], protein secretome profiles of a handful of fungi (EMSL user project, [9]), and analyses of the phylogenetic distribution of key biogeochemical pathways [10].

The SFA has applied targeted metagenomics to 13 ecosystem studies and shotgun metagenomic techniques to 6 studies to assess the degree of community activity in response to different perturbations, and to identify the taxa and potential functions most affected (Q1 report and Table 1). Targeted metatranscriptomics (e.g., ribosomal RNA and cellobiohydrolase RNA) was used in a similar way with 5 field studies, but also improved the identification of physiologically active taxa/genes at the time of sampling [11, 12]. To expand this approach, a shotgun metatranscriptomic technique was developed and applied to broadly examine actively expressed genes in soils [3, 13]. The SFA also leveraged a form of targeted proteomics (eco-enzyme assays) to quantify the activity level of a few specific microbial functions [14-16]. The suite of techniques provides hierarchical data, ranging from organism-specific *potential* activity for all metabolic functions (metagenomics) to community-level *realized* activity for a few key functions (Eco-Enzyme Assays). These techniques were generally consistent in showing activity patterns that varied in complex ways with soil depth and ecosystem [2, 4, 17-20].

Building on prior experience, the SFA now has a revised strategy to improve discovery of microbial processes that can drive variation in ecosystem function. Metagenomic and metatranscriptomic techniques remain central to monitoring microbial activity. However, the SFA is now integrating these techniques tightly with additional measurements (C flux, stable isotope tracing, metabolomics) and computation techniques to accelerate understanding of community composition, microbial “activity”, and ecosystem function.

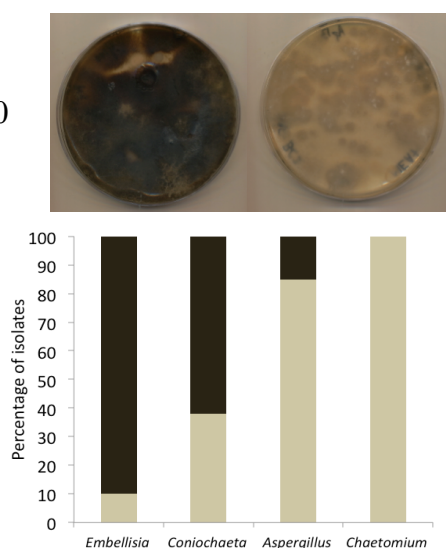
## Background

Microbial “activity” in environmental samples has different meanings depending on the ‘omics’ technique used and/or how the data are analyzed. At the coarsest level, microbial activity represents the effect-size of a perturbation at a community level. Greater community dissimilarity reflects a greater degree of microbial activity that may contribute to functional divergence. The measurements of community-level activity are linked to “who” is most active in the sense of being responsive (negatively or positively) to a perturbation and how many are responsive. This provides a stepping stone to the more difficult question, “what are they doing”, if strong links between taxon identity and function are known (e.g. cyanobacteria are primary producers). The functional aspect of “activity” (i.e. “what are they doing”) at the protein, cellular, and community level remains the most difficult undertaking constrained by expense, technical challenges, and reference data. Reference data from pure cultures provides a crucial foundation for this endeavor. Consequently, the first sections of this report (below) describe SFA efforts to augment reference data. Functional activity has the added complexity of being delineated as “potential” versus “realized” depending on the measurement spectrum from DNA (potential) to protein isoform (fully realized) and is further divided into “cosmetic” versus “consequential” depending on relevance to altered ecosystem function.

## Reference data to inform ‘omics measurements of microbial activity in the environment.

*Fungal cultures.* SFA-funded collaborator Dr. Andrea Porras-Alfaro built a fungal collection comprising about 1,000 fungal isolates each from pine forest (NC) and arid grassland (UT) ecosystems. About 90% of the isolates were typed to genus-level by ITS sequencing, and about 70% were also typed by LSU sequencing. Four MS thesis students, two honors undergraduate students, and two student research assistants at WIU led aspects of this project. Each student focused on a specific feature of the collection, including comparison of biocrust/sub-biocrust/rhizosphere isolates and phenotyping (characterization of keratin-degrading, plant growth-promoting, entomopathogenic, and heavy metal resistance).

The following highlight illustrates how these studies inform ‘omics measurements of microbial activity in the environment. The culture collection included numerous Dothidiomycete fungi—a group that multiple arid-land fungal rRNA gene surveys documented as abundant in surface soils. Characterization of these fungi showed they are highly stratified by soil depth, and biocrust dwellers are highly pigmented whereas those below the biocrust or in plant root zones are hyaline (Figure 1).



**Figure 1.** Melanized and hyaline fungal isolates from the surface biocrust (left) and 5 cm below the biocrust (right). Lower bar graph shows the proportion of pigmented to hyaline isolates of four dominant genera. Porras-Alfaro, in prep.

*Actinobacteria culture.* Actinobacteria are very abundant in arid land soils. To advance understanding of the potential role of Actinomycetales in decomposition of complex plant substrates, the SFA developed a collection of over 100 unique isolates (by 16S rRNA typing) from arid land soil samples. The isolates represent 14 families and 30 genera. Isolates representing each of the 8 most dominant Actinomycetales families detected in the arid land soils by Illumina 16S rRNA sequence libraries were successfully cultured. Isolates were subjected to a

battery of substrate transformation assays to determine their ability to catalyze extracellular degradation of complex polysaccharides including: crystalline cellulose (Avicel, filter paper), carboxymethyl cellulose, xylan, chitin, pectin and starch. Isolates were also tested for peroxidase and laccase activity. Exopolysaccharide degradation capability (in particular hemicellulose or hemicellulose-like substrates) was widespread among the Actinomycetales, but degradation of specific substrates clearly exhibited phylogenetic clustering (Figure 2; [7]).

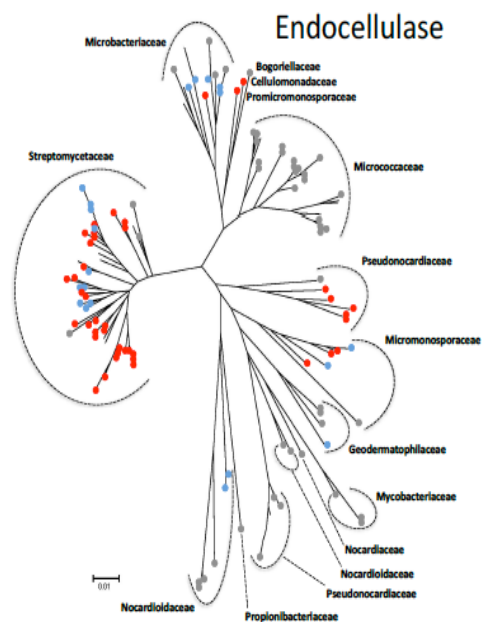
Systematic measurement of functional characteristics of isolates and development of phenotypic references databases is a vital step to improve the interpretation of microbial functional activity in environmental samples measured with ‘omics techniques.

**Reference genomes.** To improve genome reference databases, the genomes of 9 abundant or unusual fungi in the collection were sequenced and 12 others are in progress. Likewise, genomes of 6 diverse Actinomycetales and 5 other arid land bacteria were sequenced to improve coverage of poorly-represented groups in public databases.

**Reference proteomes.** Proteomic profiles of pure cultures provide useful reference data for interpretation of metatranscriptomic measurements of microbial activity in environmental samples. Proteomic data more accurately reflect “realized” function, whereas gene expression is intermediate between “potential” and “realized” function. In fact, comparisons of proteome and transcriptome data for bacteria and fungi continue to show that only a few percent (e.g., 2-4%) of differentially expressed genes are detected as differentially expressed proteins (e.g. [21]). To assess variability in extracellular proteins among fungi, the secretomes of 5 fungi on 4 substrates were measured via an EMSL user project [9]. The Ascomycota fungi were isolates from arid grassland soil samples (UT): *Coniochaeta*, *Embellisia*, *Chaetomium*, *Phoma*, and *Aspergillus* from the soil. The fungi were grown in replicate cultures on chitin, grass litter, or pine wood. The secretomes comprised a surprisingly small set of proteins from each fungus and the secretomes varied greatly among species and growth substrates [9].

**Identification of cellulose-consumers in soil via stable isotope probing (SIP).** SIP is increasingly used in tandem with ‘omics techniques to directly trace the movement of nutrients into organisms and the temporal and spatial flow. The SFA has exploited this technique only once but is poised for routine use as the program moves forward.

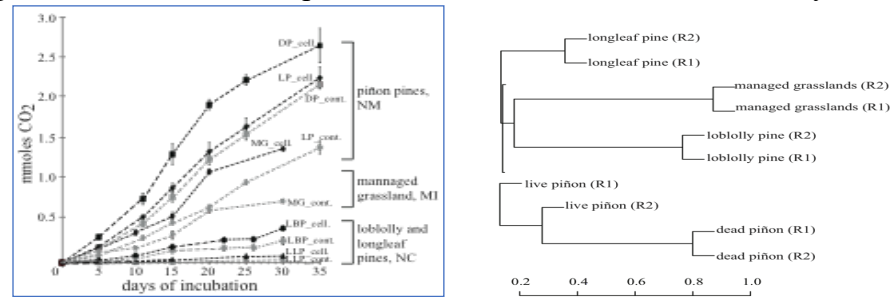
Many bacteria and fungi are known to degrade cellulose in pure culture, but their activity and response to added cellulose in soils is unknown. Replicate soil microcosms, amended with  $^{13}\text{C}$ -cellulose, were used to identify cellulose-consuming bacteria and fungi in five geographically and edaphically different soils (Figure 3, [1]). Diversity and composition of the cellulose-consuming communities was assessed by DNA-stable isotope probing, combined with sequencing of the 16S and LSU rRNA genes for the bacteria and fungi, respectively. In each soil,



**Figure 2.** Phylogenetic clustering of endocellulase activity among ~100 Actinobacteria isolates. Red dots – high activity; blue dots – moderate activity; grey dots 0 no activity.

the  $^{13}\text{C}$ -enriched, cellulose consuming guilds differed from the composition of the original soil community or  $^{12}\text{C}$ -labeled controls. Although similarities of higher-level taxa occurred among some soils, the composition of cellulose-consuming bacteria and fungi was generally specific for each soil type, suggesting either historical or edaphic factors constrained each community.

Figure 3. Stable isotope probing to identify cellulose-responsive fungal and bacterial (not shown) communities. Left panel -  $\text{CO}_2$  efflux from soil microcosms. Right panel - compositional similarity of cellulose-consuming fungal guilds identified in the five soils (from [1]).



### ‘Omics techniques to measure microbial activity in the environment.

The ‘omics techniques used in the SFA to describe microbial activity at different levels in the environment are summarized in Table 1. Some highlights are described in the sections below.

Table 1. ‘Omics-based techniques applied in the SFA to collaborator field studies.

Ecosystem	Location	Main Factor	Metagenome		Metatranscriptome		Metaproteome	SFA Ref.s
			<sup>a</sup> target	<sup>b</sup> all	target	all	<sup>c</sup> target	
Estuary	MD	$\text{CO}_2$	+ <sup>c</sup>					[4, 5, 17]
Forest	FL	$\text{CO}_2$	+ <sup>c</sup>					[4, 5, 17]
Forest	WI	$\text{CO}_2$	+ <sup>c</sup>					[4, 5, 17, 22]
Forest	TN	$\text{CO}_2$	+					[4, 5]
Desert	NV #1	$\text{CO}_2$	+ <sup>c,d</sup>					[4, 5, 17, 23]
Forest	NC	$\text{CO}_2$ , N	+ <sup>c</sup>	+	+ <sup>c</sup>	+	+	[2, 4, 5, 12, 17, 19, 24-26]
Grassland	UT #1	N	+	+				[13]
Grassland	UT #2	N	+	+		+		[13, 18, 27-29]
Desert	NV #2	N	+				+	[15, 30]
Forest	MI #1	N	+	+	+	+		[3, 11, 31]
Forest	MI #2	N	+	+	+	+		[3, 11, 31]
Forest	MI #3	N	+	+	+	+		[3, 11, 31]
Forest	MI #4	N	+	+	+	+		[3, 11, 31]

<sup>a</sup>“target” refers to a targeted ‘omics technique in which a specific gene or transcript is examined. Targeted metagenomic and metatranscriptomics used ribosomal RNA genes or transcripts, respectively.

<sup>b</sup>“all” refers to a shotgun approach in which everything is measured simultaneously.

<sup>c</sup>The fungal cellobiohydrolase gene *cbhI* (or its transcripts) was an additional target.

<sup>d</sup>The bacterial nitrogen fixation gene *nifH* was an additional target.

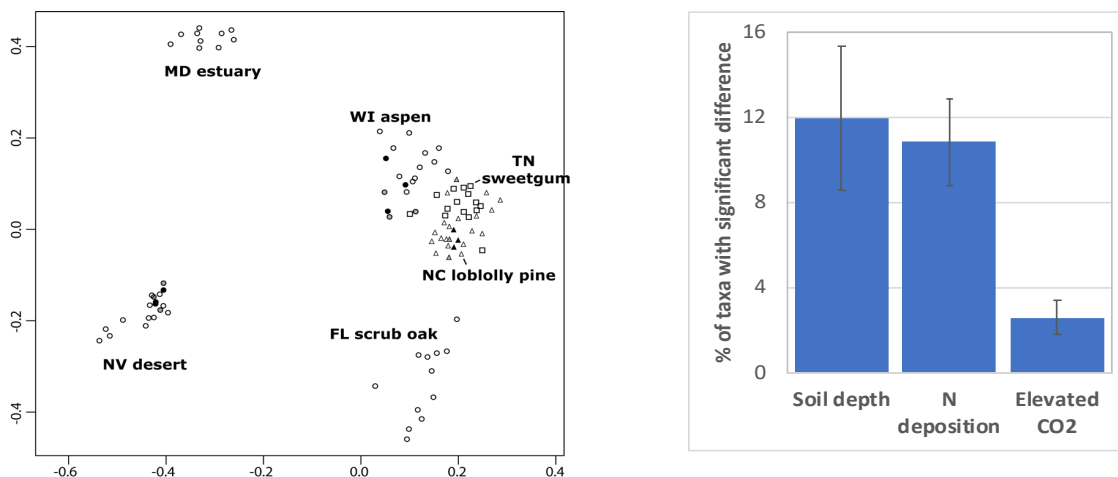
<sup>e</sup>In this table, targeted proteomics refers to use of Eco-Enzyme Assays that measure the activity of several enzymes ( $\beta$ -1,4-glucosidase, alkaline phosphatase, alanine aminopeptidase, and  $\beta$ -1,4-N-acetylglucosaminidase) [14-16] following the method of [32].

<sup>f</sup>The references are prime examples, not a comprehensive list of all publications arising from each study.

### Community-level microbial activity – ranking perturbations

Through collaborations with researchers leading field studies, the SFA has primarily examined microbial community activity in response to elevated atmospheric CO<sub>2</sub> or elevated nitrogen. However, the variety of field experiments also included other physico-chemical drivers that might shape microbial community composition and function. Drivers included geographic location, primary producer type (e.g. type of biological soil crust versus type of plant), soil parent material, soil depth strata, elevated ozone, elevated temperature, physical disturbance of soil cover, and altered precipitation patterns. Field studies were a mix of single or multi-factor experiments. The SFA did not attempt to systematically rank all of the factors in terms of their strength of impact on soil community activity, but two examples below highlight this type of activity assessment and illustrate it's potential to shape research investments.

The SFA's first major study examined microbial responses to a decade of elevated atmospheric CO<sub>2</sub> in six ecosystems. The decade-long ecosystem studies had already shown large increases in plant productivity under elevated CO<sub>2</sub>. The SFA profiled bacterial and fungal community composition at the end of the experiments by targeted metagenomic sequencing of bacterial 16S rRNA genes [4] and fungal large subunit (LSU) rRNA genes. Fungal cellobiohydrolase genes (*cbhI*) were also profiled in five of the ecosystems to specifically examine cellulolytic fungi in greater depth [5]. The composition of bacterial, fungal communities clustered by ecosystem type, as expected, (Figure 4, left panel). Of greater interest was the weak bacterial community response under elevated CO<sub>2</sub>—on average only 3% of the bacterial community showed a significant change—whereas nitrogen fertilization drove a much larger fraction of bacterial community changes. Nitrogen fertilization was also a stronger driver of change in nitrogen-fixing bacterial communities (diazotrophs) compared to long-term elevated atmospheric CO<sub>2</sub>, measured by targeted metagenomic sequencing of nitrogenase dehydrogenase gene (*nifH*) genes [17]. These observations combined with results from other researchers indicating a strong role of nitrogen deposition as a perturbation of soil microbial community activity led the SFA to make nitrogen deposition the dominant focus of the program.

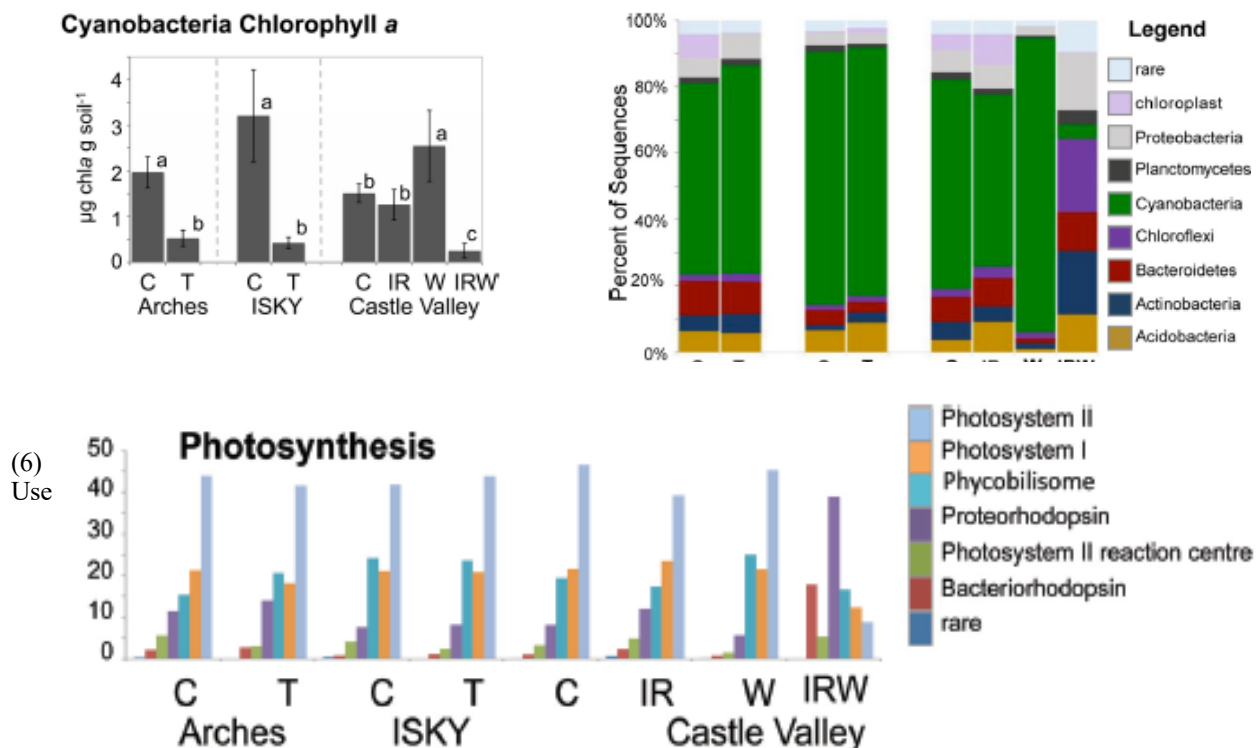


**Figure 4.** Drivers of bacterial community composition (“activity”) in six elevated atmospheric CO<sub>2</sub> field studies. Left panel - Nonmetric multidimensional scaling of Bray-Curtis values computed from OTU<sub>97</sub> presence/absence profiles of 97 16S rDNA clone libraries. Stress value = 13.9. Similar results were obtained using relative abundance instead of presence/absence data [4], or using fungal data instead of bacterial data (unpublished) or using cellulolytic fungal community data [5]. Right panel – Ranking of soil depth, elevated N fertilization, and elevated atmospheric CO<sub>2</sub> in driving bacterial community changes within ecosystems.

A combination of targeted (bacteria only) and shotgun metagenomics was used to rank the activity of fragile biological soil crusts at 3 arid grassland sites in Utah in response to physical disturbance (trampling) and climate change perturbations predicted for the region by multiple climate change models—slightly elevated temperature, altered rainfall pattern, or a combination of elevated temperature and altered rainfall [27]. Biocrusts are key to reducing erosion and providing major nutrient inputs in aridland ecosystems. This set of field experiments was led by U.S. Geological Survey collaborators, Drs. Jayne Belnap and Sasha Reed.

By visual inspection and by chlorophyll measurements, physical disturbance and the combination of heat with altered rainfall had similar destructive effects on biocrusts (Figure 2, top left panel). Important new insights arose from ‘omics measurements of bacterial community activity. Although trampling substantially reduces surface soil microbial biomass, taxonomic profiling showed that it didn’t have a major effect on the composition of bacteria at the phylum level. In contrast, the combination of heat with altered rain dramatically changed the bacterial community composition (Figure 2, top right panel).

Augmenting the study with shotgun metagenomics revealed a similar, dramatic shift in the composition of photosynthetic proteins under the heat with altered rain treatment (Figure 5, top right panel), suggesting different metabolic functional potential. This study had important policy implications. It demonstrated the flaw of using only visual inspection of biocrust cover to rank arid-land perturbations. Perturbations causing equal visual damage to soil cover can cause extremely different soil microbial changes; divergent microbial outcomes may affect the time required for restoration or cause other unanticipated ecosystem effects.



Functional activity – which taxa are active and what are they doing?

**Figure 5.** Effects of physical disturbance or climate change factors on arid grassland biocrusts. Treatments symbols are as follows: C – control; T – trampled; IR – 2°C increase in temperature; W – increased frequency of rainfall but lower quantity per event; IRW – combination of heat with altered rainfall.

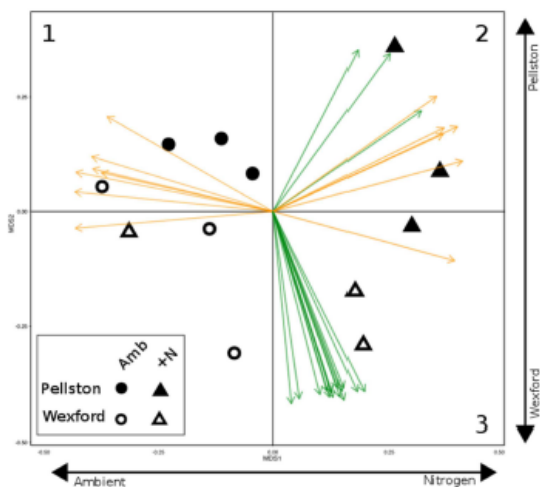


### Microbial functional activity in response to N deposition

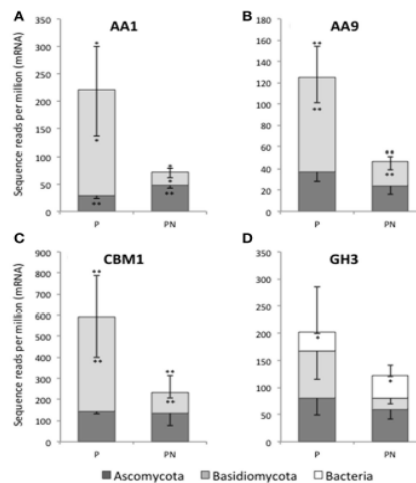
To augment the metagenomic approaches, the SFA developed a metatranscriptome technique to broadly examine gene expression in soils [3]. Prior to developing this approach, metatranscriptome studies of soil microbial communities had been difficult and rarely reported, largely because the high abundance of rRNA overwhelmed mixed-template sequencing. Our approach significantly improved upon earlier studies by (a) removing the majority of rRNA from an environmental RNA sample without relying on polyA enrichment, and thus (b) retaining the bacterial, archaeal, and eukaryote components. This approach is applicable to many studies of interactive metabolism and signaling between eukaryotes and bacteria; for examples, between plants and bacterial pathogens, or among plants, their mycorrhizal fungi and rhizosphere bacteria [3].

The metatranscriptome approach has been applied to five forest ecosystems and 1 arid grassland ([3, 11, 13], unpublished data). The example provided here compares Carbohydrate Active Enzymes in metatranscriptomes from two Maple forest ecosystems 145km apart subjected to chronic nitrogen amendment for 21 years [3]. An average of 10.6M mRNA sequence reads were obtained per sample. On average, 7% of the nearly 130M mRNA sequence reads had a significant match to a cluster in the UniRef50 protein database. Similar proportions of reads had matches to a database of KEGG motif-containing proteins. Across all samples, 0.71% of the reads could be attributed to CAZyme families (about 74, 000 reads per sample). There were more than twice as many CAZyme and UniRef50 reads attributed to eukaryotes than to bacteria.

Ordination of samples derived from the total set of CAZyme family abundances revealed shifts in expressed CAZyme patterns with site and N amendment. The CAZymes that declined under N amendment were mostly of eukaryotic origin and represented many fungal lingo-cellulolytic activities. These data are consistent with the “N-mining” hypothesis, which speculates that fungi spend energy deconstructing lignocellulose in order to obtain N, and the fungi will stop doing so if less costly sources of N are available. The CAZymes that increased



**Figure 6.** Non-metric multidimensional scaling ordination of field sample metatranscriptomes based on CAZy metranscript composition. Vectors denote highly significant correlation with ordination axes ( $p < 0.001$ ). Orange vectors - eukaryotic CAZymes. Green vectors - bacterial CAZymes. From [3].



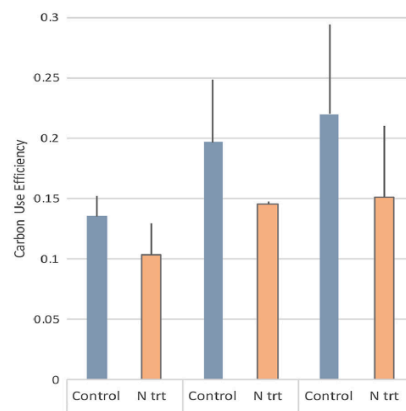
**Figure 7.** Fungal phylum-level distribution of select CAZyme transcripts, illustrating differences in expression levels between ambient (P) and N-amended (PN) metatranscriptomes. AA1 – laccase. AA9 – polysaccharide monooxygenase. CBM1 – cellulose binding domain. GH3 – broad substrate activity. From [3]

under N amendment represented a variety of functions with no unifying ecological trend. Of the 100 most abundant CAZyme families, 15 families contained both bacterial and fungal representatives. The transcript responses to N varied between bacterial and fungal members of those families; in some cases both bacterial and fungal transcripts increased, decreased, or had differential responses to N (data not shown). At the Pellston site, which exhibited the strongest microbial activity in response to elevated N, the relative abundance of CAZymes from Basidiomycota fungi declined (Figure 7, [3]), as seen in many other ecosystems under N amendment.

This highlight provides a strong example of the power of metatranscriptomics to provide coherent mechanistic insights (example, consistency with the N-mining hypothesis). A key to the success of this case was the strength of the CAZyme reference database, which provided unambiguous attribution of the source of many of the CAZymes and also the functional role. This particular study as described interesting co-expression patterns of some bacterial CAZymes that contrasted with the functional roles of a set of co-expressed fungal CAZymes, pointing to a capacity for metatranscriptome data to illuminate the relative roles of bacteria and fungi in a central ecosystem process - litter decomposition.

Metagenomic and metatranscriptomic analyses are currently underway to examine horizon-specific microbial activity associated with litter decomposition in a pine forest ecosystem (NC; Kuske in prep).

Attempts to achieve a coherent view of microbial activity in the environment by integrating metagenomic, metatranscriptomic, and a form of targeted proteomics (Eco-Enzyme Assays; EEAs) are complicated and ongoing [2]. EEAs have been successful as an orthologous technique to investigate microbial carbon use efficiency in environmental samples, and has documented patterns of microbial activity in response to nitrogen loading rates across diverse ecosystems and biomes [2, 14-16]. The effectiveness of EEAs in discerning common patterns of microbial activity among ecosystems [14-16] suggests that similar achievements should be possible with broader ‘omics techniques. The ongoing challenge is to understand how to cluster different types of ‘omics data into the right functional bins that provide the measurements of “realized” microbial activity in the environment needed to discover key processes.



**Figure 8.** Mean microbial carbon use efficiency (CUE) by horizon and treatment  $\pm$  95% confidence intervals. CUE varied significantly by horizon and decreased significantly with N amendment. From [2]

### Shift in SFA strategy

The SFA developed a new strategy to overcome the challenges widely encountered when applying ‘omics techniques to ecosystem studies. The basic challenge is that documenting microbial activity in response to perturbations is fairly easy with ‘omics techniques, but identifying which activities matter and are not already captured by existing variables in soil carbon models is extremely difficult.

Our new strategy emphasizes discovery of microbial communities that represent substantially different (and measurable) functional states *under the same environmental conditions*, not perturbed versus unperturbed conditions. Table 2 summarizes ‘omics techniques



used in the SFA to examine microbial activity in initial experiments that address the new SFA strategy.

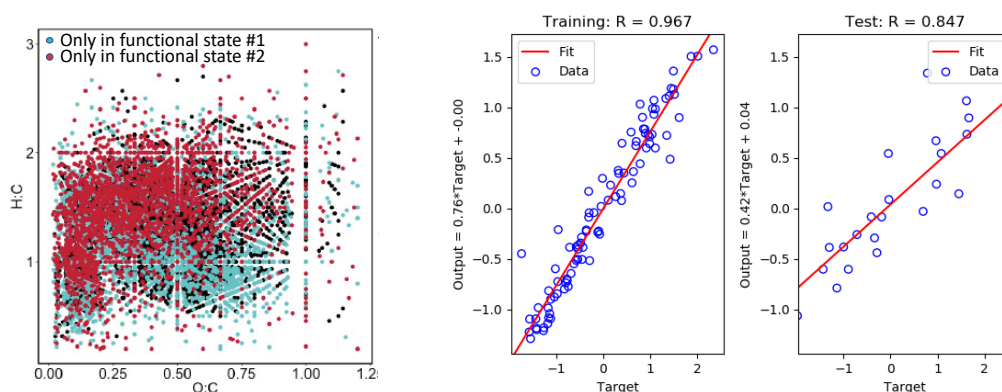
Table 2. ‘Omics-based techniques applied thus far in new phase of SFA.

Source soils	Total ucosms Microcosms	CO2	Dissolved Organics	M-genome t <sup>a</sup>	M-transcript s <sup>b</sup>	Metabolomic s
209	618	+	+	+	+	+
100	200	+	+	+	+	+
100	200	+	+	+	+	+

<sup>a</sup>“target” refers to a targeted ‘omics technique in which a specific gene or transcript is examined. Targeted metagenomic and metatranscriptomics used ribosomal RNA genes or transcripts, respectively.

<sup>b</sup>“all” refers to a shotgun approach in which everything is measured simultaneously.

Incorporation of a different types of metabolomics has been especially informative and has enabled new types of analyses that are already providing insights relevant to goals of the program. Figure 9 illustrates the rich metabolomic data acquired through an EMSL user project and shows substantial differences in the composition of dissolved organics acquired from microbial communities that represent different two contrasting functional states in plant litter decomposition. Machine learning and other analytical techniques provide a means to infer microbial taxa that control the abundance of specific compounds, providing insights into underlying physiological and ecological mechanisms.



**Figure 9.** Metabolomic data from >300 plant litter decomposer communities. Left panel – Van Krevelen plot of chemical composition. Right panels – Machine learning model using metagenomics data to predict the abundance of a particular chemical component Ulrich in prep.

Within the new SFA strategy, metagenomic and metatranscriptomic techniques remain central to monitoring microbial activity. However, the SFA is now integrating these techniques tightly with additional measurements (C flux, stable isotope tracing, metabolomics) and computation techniques to accelerate understanding of community composition, microbial “activity”, and ecosystem function.

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